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Advances in Phytase Research

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recombinant strain of *H. polymorpha* (Mayer *et al.*, 1999). This system featured the use of an economical carbon source, glucose or glucose syrups, as a means to make this a low-cost process. However, lower yields (6.1 g/liter) were obtained when the *A. fumigatus phyA* gene was expressed in the system.

C. Expression in Plants

The gene for fungal phytase has been successfully overexpressed in several transgenic plants (Day, 1996; Verwoerd *et al.*, 1995; Li *et al.*, 1997). The ability to express recombinant phytase in plants offers the possibility for the development of plant varieties that would contain sufficient amounts of phytase in their grain or seed so that phytase supplements would not be required. In addition, the potential use of crop plants to serve as bioreactors to produce phytase commercially is being investigated. In 1995, Verwoerd and coworkers in Holland did express a functional phytase in *Nicotiana tabacum* through a constitutive expression of phytase cDNA and showed that the enzyme was secreted out of the cells. They achieved secretion to the extracellular fluid by the use of a signal sequence from the tobacco pathogen-related protein S (Verwoerd *et al.*, 1995). The expressed phytase was found to be biologically active and accumulated in leaves up to 14.4% of total soluble protein during plant maturation. Researchers at the University of Wisconsin Biotechnology Center have independently expressed the *A. niger* NRRL 3135 *phyA* gene in tobacco leaves. The full 441-aa protein was made in leaf tissue, which was purified to homogeneity and extensively characterized (Ullah *et al.*, 1999). Except for a decrease in molecular mass due to reduced glycosylation, the expressed recombinant phytase was virtually the same as native fungal phytase. The catalytic properties of the cloned phytase were encouraging enough to open the possibility of overexpressing the fungal phyA gene in other crop plants. This could pave the way for producing phytase commercially in field crops.

University of Wisconsin researchers have also developed alfalfa plants to commercially produce phytase. They performed cloning and expression of the fungal phytase gene, so that most of its product was contained in the juice collected after the alfalfa was processed (Gutknecht, 1997). The equipment investment for this biofarming process is minimal and potentially turns a byproduct into a source of additional income for the farmer. Other enzymes have also been expressed in these

plant "bioreactors." But the results achieved with phytase enhances the feasibility of future development of this technology to produce this enzyme commercially.

Another application of biotechnology in plants is to reduce the need for phytase by lowering phytate levels in the plant's cereal or meal. Maize cultivars with reduced levels of phytic acid have already been produced (Raboy and Gerbasi, 1996). Transgenic soybean isolates that overexpress fungal phytases and thus eliminate or reduce the need to supplement meal with phytase are also being pursued. Li *et al.* (1997) have expressed the *A. niger* NRRL 3135 phytase gene in soybean (*Glycine max*). The recombinant phytase had a lower molecular weight than the native fungal enzyme, but its temperature and pH optimum were almost identical to that of the native enzyme.

This strategy of having transgenic plant overexpressed phytase still requires a heat-tolerant phytase to survive the elevated temperatures often required in feed production. Recent work at the Swiss Federal Institute of Technology in Zurich details the transfer of a heat-tolerant *A. fumigatus* phytase gene by transformation and its expression in rice (*Oryza sativa* L.). This research is targeted at improving the nutritional profile of rice by reducing the amount of phytate. Phytate binds up to 95% of the iron in rice and keeps it from being absorbed. Therefore, individuals in parts of the world with a high-rice diet are prone to iron deficiency (Gura, 1999).

D. Transgenic Animals

In the future, transgenic poultry, hogs, and so on may produce phytase in their own digestive tract. Several attempts have already been made to transform and express a fungal phytase in an animal (privileged information, personal communication). To date none of these attempts have been successful. Similar results were obtained when the *phyA* gene was expressed in *E. coli* (Phillippy and Mullaney, 1997). The problem could very well be associated with glycosylation or its lack in animal and bacterial cells, respectively. Native fungal phyA protein contains 10 asparagine (Asn) residues with glycosylation signals that are all *N*-glycosylated. In *E. coli* these Asn residues are not glycosylated, and perhaps the recombinant protein does not fold appropriately to produce active site geometry, which is essential for activity. Similarly, in animal systems where *O*-glycosylation is preferred, the 10 *N*-glycosylation sites will be left unglycosylated. This may explain the lack of activity of phyA protein expressed in animals. Perhaps engi-

neering *O*-glycosylation sites at these locations may allow the mammalian cells to glycosylate either the threonine or serine residues and thereby allow for appropriate folding of recombinant phytase. This, however, needs to be tested.

As more is understood about the structure–function relationships of the microbial phytases, another possible avenue is to modify an animal's HAP, that is, rat acid phosphatase (Kostrewa *et al.*, 1997; Schneider *et al.*, 1993), or multiple inositol polyphosphate phosphatase (Craxton *et al.*, 1997) to enhance its ability to hydrolyze phytin. If successful, this would reduce the difficulty of obtaining expression in animal tissue.

VII. Expanding Uses of Phytase

A. Potential in Aquaculture

Numerous studies have been conducted on the use of soybean meal or other plant meals in aquaculture, including feeding studies on rainbow trout (Watanabe and Pongmaneerat, 1993; Mwachireya *et al.*, 1999), the greenback flounder (Bransden and Carter, 1999), and the African catfish (van Weerd *et al.*, 1999). By substituting lower-cost plant protein for a more expensive protein source, such as menhaden fish meal, a significant cost reduction could be achieved. Feed costs constitute up to 70% of total fish production costs (Rumsey, 1993). The consumer price index over the period of 1982–92 showed that, while the price index for seafood increased by more than 50%, the cost of alternative proteins increased only an average of 30% (Chamberlain, 1993).

As in poultry and hogs, fish lack an adequate digestive enzyme to effectively utilize the phytin phosphorus in this feed. Moreover, as aquatic animals, the problems associated with high phosphorus levels in the water from their waste is an immediate problem. Therefore, phytase has been evaluated as a means to both increase the use of low-cost plant meals in the aquaculture industry, and also to maintain acceptable phosphorus levels in the water. Several fish feeding studies have documented the potential value of phytase in diets containing high levels of plant feedstuffs (Robinson *et al.*, 1996; Oliva-Teles *et al.*, 1998: Mwachireya *et al.*, 1999).

Chamberlain (1993) has projected that global seafood consumption will increase 35% by 2025. The aquaculture industry will supply an increasing amount of the world's need for seafood. Whereas the major market for phytase today is as a food additive in poultry and hog feed, there is great potential to expand the market into feedstuffs used in aquaculture. The higher temperature required for pelletization of feed in aquaculture and the lower body temperature of fish may require the development of commercial phytases tailored for aquaculture feeds.

B. Phytase as a Soil Amendment

In certain locations, phytic acid and its derivatives may represent up to 50% of the total organic phosphorus in the soil (Dalal, 1978). This abundance of phytic acid in the soil and the possibility that the addition of phytase might stimulate plant growth in these soils has been investigated. Findenegg and Nelemans (1993) studied the effect of phytase (phyA) on the availability of phosphorus from phytic acid in the soil for maize plants. Growth stimulation was reported as the result of an increased rate of phytin hydrolysis when phytase was added to the soil. However, the amount of phytase necessary for a significant effect meant that this was not a practical technique at this time. This study also suggested that the expression of phytase in the roots of transgenic plants might increase the availability of phosphorus to plant roots (Day, 1996). To better understand the role root phytase plays in the phosphorus nutrition of plants, the phytase and acid-phosphatase activity of extracts from several temperate pasture grass and legumes were isolated and studied (Hayes *et al.*, 1999).

C. Production of *myo*-Inositol Phosphates

Greiner and Konietzny (1996) investigated the use of *E. coli* phytase to generate specific breakdown products from phytic acid. A packed-bed bioreactor containing covalently attached *E. coli* phytase was constructed in this study to economically produce special isomers of the lower *myo*-inositol phosphate esters. The bioreactor chiefly yielded $I(1,2,3,4,5)P_5$, $I(2,3,4,5)P_4$, $I(2,4,5)P_3$, and $I(2,5)P_2$ isomer forms. Because only one major isomer of each *myo*-inositol phosphate species was formed, further purification could be easily achieved by ion-exchange chromatography.

The K_m for phytate increased from 130 μM for free enzyme to 240 μM when *E. coli* phytase was immobilized. However, the catalytic turnover number was lowered from 6209 per second for free phytase to 1182 per second. Thus, on crosslinking, *E. coli* phytase's catalytic activity was slowed down drastically. However, the immobilized bacterial enzyme performed much better than the immobilized *A. niger* NRRL 3135 phyA (Ullah and Cummins, 1988; Dischinger and Ullah, 1992). One reason